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Secretion of Lysosomal and Digestive Enzymes into Pancreatic Juice under Physiological and Pathological Conditions in Rabbits

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Abstract

To investigate the possible secretion of lysosomal enzymes into pancreatic juice during stimulation with a pancreatic secretagogue under both physiological and pathological conditions, we measured the amount of cathepsin B, a lysosomal enzyme, in the pancreatic juice during the infusion of 6 different concentrations of caerulein (0.02, 0.05, 0.2, 0.5, 1.0, and 2.0 μg/kg. hr). In one group of rabbits the pancreatic duct was only cannulated (free-flow group); in others the pancreatic duct was obstructed for 7 hours and secretin was infused at 0.2 CU/kg. hr (obstructed group). In addition, we evaluated the effect of the intraduodenal instillation of a liquid meal (2 g/kg) on the secretion of lysosomal enzymes into pancreatic juice.

Caerulein stimulated the secretion of cathepsin B into pancreatic juice in a dose-dependent manner, as it did that of amylase, and at higher concentrations of caerulein (1.0 and 2.0 μg/kg. hr), both cathepsin B output and amylase output were decreased. There was a significant positive correlation between cathepsin B output and amylase output into pancreatic juice during stimulation with caerulein. Blockage of the pancreatic duct for 7 hours caused a significant rise in serum amylase levels and a redistribution of cathepsin B activity in the pancreatic subcellular fractions, as a result of which an increased amount of cathepsin B was recovered in the pellet obtained by 1000× g centrifugation for 15 min, which contained many zymogen granules. These changes noted after short-term pancreatic duct obstruction are very similar to those previously noted in the early stage of diet-and caerulein-induced experimental pancreatitis, suggesting the colocalization of lysosomal enzyme and digestive enzymes.

In the duct-obstructed animals, the secretion of cathepsin B stimulated by caerulein was significantly greater than in the free-flow group.

Furthermore, the intraduodenal instillation of a liquid meal caused the secretion of cathepsin B

Key words: Amylase, Lysosomal enzyme, Cathepsin B, Caerulein, Pancreatic duct obstruction
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into the pancreatic juice along with amylase.

These results indicate that under physiological conditions, such as food intake, lysosomal enzymes are secreted into the pancreatic juice in response to stimulation by gut hormones in the same manner as classical pancreatic digestive enzymes. Moreover, zymogen colocalized with lysosomal enzymes in duct-obstructed animals is secreted into pancreatic juice in increased amounts together with digestive enzymes; this finding suggests that lysosomal enzymes play important pathophysiological roles in pancreatic juice and that acinar cells are altered to maintain cellular organization by secreting the potentially dangerous lysosomal enzymes. This pancreatic duct-obstructed rabbit model should be useful in clarifying the early events of acute pancreatitis.

Introduction

Both pancreatic digestive enzymes and lysosomal hydrolases are synthesized in ribosomes attached to the endoplasmic reticulum, then co-transported to the Golgi complex where lysosomal hydrolases are glycosylated with mannose-6-phosphate and bound to mannose-6-phosphate specific receptors, and lysosomal enzymes are transported to lysosomes[18,33,38]. The digestive enzyme zymogen is packaged in condensing vacuoles which mature into zymogen granules as they migrate towards the luminal plasmalemma, where they discharge their contents into the ductal space[27]. Thus, these two enzymes are separated in the normal physiological state in pancreatic acinar cells for the maintenance of normal cellular organization, because the mixture of these two enzymes is potentially dangerous to the acinar cells. Although there would seem to be alomost no possibility for lysosomal enzymes to be secreted into pancreatic juice when stimulated by pancreatic secretagogues, there have been some reports of secretory profiles of lysosomal enzymes in many cell lines[11,15,20,44], and of their presence in pancreatic juice[32]. The possible secretion of lysosomal enzyme into pancreatic juice under normal conditions and in patients with chronic calcifying pancreatitis has also been reported[10]. These studies suggest that lysosomal enzymes have both physiological and pathophysiological significane in biological fluids[19]. Most of these reports, however, described in-vitro studies, and the few in-vivo studies were not very systematically or quantitively performed, so the possibility of contamination with duodenal juice or bile and the effect on the exocrine pancreas of endoscopic catheterization of the pancreatic duct could not be ruled out. Therefore, the possible stimulation by pancreatic secretagogue of the secretion of lysosomal enzymes remains to be elucidated. Both morphological and biochemical studies have shown that, in the early stage, two forms of experimental pancreatitis (diet-induced[16,25], and secretagogue-induced[34–36,42]), as well as a model of experimental pancreatitis in pancreatic duct-obstructed rabbits[37], share the common attribute of co-localization of digestive enzymes with lysosomal hydrolases inside large cytoplasmic vacuoles[39] and redistribution of lysosomal enzymes from the lysosome-rich to the zymogen granule-rich fraction. Since cathepsin B, a lysosomal enzyme, can activate trypsinogen[9,13,14,31], and trypsin can activate the other pancreatic digestive enzymes, the colocalization of digestive enzymes with lysosomal hydrolases could lead to the activation of intracellular digestive enzymes associated with increased lysosomal fragility and be an important trigger in the development of acute pancreatitis inside the acinar cells.

Thus, lysosomal enzymes seem to play an important role in the pathogenesis of acute pancreatitis[40]. In contrast to the reversibility that characterizes these secretagogue-induced and rabbit pancreatic duct-obstructed model of mild pancreatitis, diet-induced pancreatitis may be characteriz-

ed by hemorrhagic necrosis of the gland. Furthermore, it is not clear that the results of panncreatic duct obstruction can be extrapolated to the situation in clinical pancreatitis, since the clinical disease is obviously not induced by ethinonine ingestion and is unlikely to be the result of supramaximal secretagogue stimulation. On the other hand, gallstone pancreatitis, which is the most common form of acute pancreatitis in humans, seems to be triggered by the passage of a stone or its incarceration in the terminal bile duct. In has been suggested that such a stone might obstruct the pancreatic duct, but the mechanism whereby pancreatic duct obstruction induces pancreatitis has not been clarified. Our model of pancreatic duct obstruction in rabbits seems to have many advantages in the investigation of the mechanism of duct obstruction in pancreatitis, because the pancreatic duct is completely separate from the biliary system in rabbits, and the effects of bile or biliary obstruction can be completely ruled out.

In this study, we examined the secretion of lysosomal enzymes into pancreatic juice during stimulation with a pancreatic secretagogue or after intraduodenal liquid meal was instilled under normal conditions and shortly after obstruction of the pancreatic duct. The possible secretion of digestive enzymes colocalized with lysosomal enzymes during stimulation with secretin and caerulein was also investigated.

Materials and Methods

New Zealand White rabbits of both sexes weighing 2.20 to 2.95 kg (Shizuoka Experimental Animals, Shizuoka, Japan) were used in this study.

They were kept in light-dark cycle regulated (5:00–17:00) and air conditioned (23±3°C) animal quarters in our hospital before the experiments. Thests were performed after a 6-hour fast starting at 9:00 AM to rule out the effects of circadian rhythm on the exocrine pancreas.

In-vivo secretion of lysosomal enzymes into pancreatic juice during stimulation with a pancreatic secretagogue in normal rabbits

Anesthesia was induced by the intravenous adiministration of sodium pentobarbital (35 mg/kg) through an ear vein and maintained by periodic intravenous injections of pentobarbital (10 mg/kg).

Animals were kept on heating pads at 40°C (American Medical Systems, Cincinnati, OH, U. S. A.) to maintain body temperature. Before opening of the abdomen, a catheter (PE 50, Clay Adams, Parsippany, NJ, U. S. A.) was paused through the right femoral vein into the inferior vena cava. A midline skin incision was made from the xiphoid process to the unbilicus, the pylorus was ligated, and a catheter (PE 70) was inserted through a gastrostomy to draw off gastric juice. Another catheter (PE 10) was inserted into the pancreatic duct just adjacent to its opening into the duodenum for a distance of 5 mm, and heparinized saline (30 U/ml) was infused continuously at a speed of 1.58 ml/hr with an infusion pump (Harvard Apparatus, Millis, MA, U. S. A.). The external portion of the catheter in the pancreatic duct was placed on a sponge soaked with warm saline and coverd with gauze to keep it wet and warm. The position of the catheter could therefore be checked constantly.

The rest of the abdominal wound was closed and also covered with gauze soaked in warm saline. In addition, the animals were kept warm by lights from above during the experiment, and the covering gauze was sprinkled with warm saline every hour. After a 30 min period of stabilization, all animals were infused with caerulein (Sigma Chemical Co., St. Louis, MO, U. S. A.) in 6 different concentrations (0.02, 0.05, 0.2, 0.5, 1.0 and 2.0 μg/kg. hr) at a speed of 1.58 ml/hr for 3 hours to stimulate the secretion of digestive enzymes. Pancreatic juice was collected hourly (frac-

tions C_1, C_2, and C_3) in eppendorf tubes on ice, and the volume of pancreatic juice was determined by direct weighing on an automatic electronic balance, substracting of the tube weight and adjusting the density of the juice to 1.0 g/ml. Amylase activity in each fraction was measured by the method of Bernfeld[4] with soluble starch as the substrate, and 1 unit (U) of amylase activity was defined as that which releases 1 mg of maltose per min from the substrate. As a lysosomal enzyme, cathepsin B activity was measured fluorometrically by the method of McDonald and Ellis with CBZ-2-arginyl-arginine-β-naphthylamide (Bachem Bioscience, Philadelphia, PA, U. S. A.) as the substrate[23], and 1 unite (U) of cathepsin B activity was defined as that which releases 1 nanomole of β-naphthylamide (Sigma Chemical) per min from the substrate. Bothe amylase and cathepsin B outputs were expressed as U/kg. hr. All the animals used in this experiment were sacrificed by a large dose of pentobarbital, and the pancreas was used for the experiments on subcellular fractions. Immediately after collection, each fraction was placed in a small box and stored in the refrigerator until the day of examination. Cathepsin B activity was measured on the same day.

Subcellular fractionation

Pancreatic tissue was obtained from the rabbits used in the in-vivo caerulein stimulation experiment, for determination of the distribution of lysosomal enzyme in acinar cells. Excised, trimmed, and homogenized rabbit pancreas was separated into its various subcellular fractions by differential centrifugation.

The protocol originally developed by Tartakoff and Jamieson[41] with modification for studies of rat tissue[6], was further modified to permit optimum separation of rabbit pancreatic cell fractions[37]. Briefly, pancreatic fragments were homogenized in cold 0.3 M sucrose solution with three up-and-down strokes of a Dounce homogenizer (Wheaton, Milleville, NJ, U. S. A.). The resulting homogenate was centrifuged (150 × g, 10 min, 4°C) to pellet debris and unbroken cells, which were discarded. The supernatant after this low speed centrifugation was considered to contain 100% of each of the components measured. This supernatant was centrifuged (1000 × g, 15 min, 4°C) to obtain a zymogen granule-rich pellet (1.0 KP) and the supernatant was centrifuged again (12,000 × g, 12 min, 4°C) to yield a lysosome and mitochondria-rich pellet (12 KP). The supernatant was centrifuged once more (105,000 × g, 60 min, 4°C) to obtain a microsome-rich pellet (105 KP), and the final supernatant was considered to contain soluble elements (105 KS). The various pellets obtained during fractionation were resuspended individually in 2 ml of cold (4°C) 0.3 M sucrose solution, and cathepsin B activity in each fraction was measured and expressed as a percentage of the total activity. This subcellular fractionation was performed with pancreatic tissue from 4 normal, not surgically manipulated, rabbits which constituted the control group.

In-vivo secretion of lysosomal enzyme into pancreatic juice after intraduodenal instillation of a liquid meal in normal rabbits

Following a 16 hours fast, 5 rabbits were anesthetized and a femoral vein was catheterized as described above. All the experiments were started at 9:00 AM in order to rule out the effects of circadian rhythm on the rabbit exocrine pancreas. The abdomen was opened , the pylorus was ligated, and a catheter (PE 205) was inserted into the stomach to drain off the gastric juice. Another catheter (PE 160) was inserted into the descending duodenum to infuse the liquid meal. The pancreatic duct was catheterized as described above for the collection of pancreatic juice. During the experiment, all the animals were given heparinized (30 U/ml) 150 mM NaCl solution at a speed of 1.58 ml/hr, and kept as described above.

After about 30 min, when the pancreatic juice flow had stabilized, a base-line sample of pan-

creatic juice (fraction Pre) was collected. Then the liquid meal (Isocal®, Mead Johnson: 15.3% protein, 19.7% fat, 59.7% carbohydrate, 2 g/kg body weight in 10 ml of water), was instilled into the duodenum through the duodenostomy catheter for 15 minutes with an infusion pump. After collection of the base-line sample six more samples of pancreatic juice (D_1–D_6) were obtained at 30 min intervals during and after infusion of the liquid meal. All the samples were placed in preweighed eppendorf tubes on ice, and the volumes of pancreatic juice were calculated by subtracting the tube weights with the density of the juice assumed to be 1.0 g/ml. The protein concentration of each fraction was measured by the method of Lowry et al[22]. Volume of pancreatic juice in each fraction was expressed as ml/kg. 30 min. Amylase and cathepsin B output was expressed as U/kg. 30 min, and protein output as mg/kg. 30 min.

Rabbit pancreatic duct obstruction model

Other rabbits, after catheterization of the pancreatic duct as described above, were continuously infused with secretin (Sigma Chemical) in a concentration of 0.2 CU/kg. hr at a speed of 1.58 ml/hr to stimulate pancreatic juice production.

Two groups of cannulated animals were studied: one with free-flowing pancreatic juice with the drainage catheter maintained in its original horizontal position and one with obstruction of the pancreatic duct (after 30 min for stabilization, the free end of the catheter was raised to a vertical position). In the latter group, pancreatic secretion reached equilibrium in the vertical positioned catheter at a level sufficient (18.5–23.5 cm) to exert a hydrostatic pressure that matched the secretory pressure of the pancreas. Blood samples (0.5 ml) were taken before and 1, 3, 5, 7 hours after obstruction or catheterization of the pancreatic duct from the catheter in the femoral vein, and serum amylase activity was measured as described above. Seven hours after obstruction or simple catheterization of the pancreatic duct, all the animals were sacrificed with a large dose of pentobarbital, and portions of the pancreas were removed and trimmed of fat. One part of the pancreas was used for quantitation of the pancreatic water content by comparison of the weight immediately after removal (wet weight) with the weight after desiccation at 150°C for 48 hours in a desiccator (Isotemp® Oven, Fisher Scientific, Fair Lawn, NJ, U. S. A.) (dry weight). Other portions of the pancrease were used for subcellular fractionations as described above, and redistribution of cathepsin B activity in the subcellular fractions was looked for. In the control group the same protocol was performed, but the animals were only infused with secretin without catheterization of the pancreatic duct.

Changes in amylase and cathepsin B secretion caused by stimulation with caerulein in rabbits with pancreatic duct obstruction

In some rabbits, the pancreatic duct was catheterzied as described above, and secretin (0.2 CU/kg. hr) was infused at a speed of 1.58 ml/hr. Three groups of animals were studied. Those with free-flowing pancreatic juice for 7 hours, then an infusion of secretin plus caerulein (0.2 μg/kg. hr) for 2 hours; those with pancreatic ducts obstructed for 7 hours, then an infusion of secretin only for 2 hours; and those with the pancreatic duct obstructed for 7 hours, then an infusion of secretin plus caerulein (0.2 μg/kg. hr) for 2 hours.

In the first, or free-flowing group, pancreatic juice was collected hourly in eppendorf tubes cooled on ice for 9 hours (fractions F_1–F_7, CS_1, CS_2), and the amylase and cathepsin B activity in each fraction was measured as described above. In the second and third groups with the pancreatic duct obstructed for 7 hours and secretin or secretin plus caerulein infused for 2 hours fractions of pancreatic juice (S_1, S_2) or (CS_1, CS_2) were collected and the amylase and cathepsin B activity in each

fraction was measured. At the end of these experiments, all the animals were sacrificed with a large dose of pentobarbital, and portion of the splenic lobe of the pancreas was removed and homogenated in 6 ml of cold phosphate buffered saline (pH 7.4) containing 0.5% triton X-100 (Fisher Scientific) in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, U. S. A.). Unbroken cells and debris were removed by low speed centrifugation (150×g, 15 min, 4°C), the resulting supernatant was used for the determination of "total splenic lobe" amylase and cathepsin B content, and the total pancreatic amylase and cathepsin B content was calculated. The amylase and cathepsin B activity in each fraction was expressed as a percentage of the splenic lobe content and as output in U/kg. hr.

Other portions of the pancreas were used for subcellular fractionation, and lysosomal enzyme redistribution was examined.

For the total pancreatic amylase and cathepsin B content, we used the splenic lobe because it can easily be removed and has only a little fatty tissue, while the other porions, such as the gastric lobe or the mesentric portion, are rich in fatty tissue, and difficult to separate quickly and completely from fat, blood vessels and lymphatic tissue.

Data presentation

The results reported in this communication represent the mean±SEM for n determinations. Deviation bars in the figures indicate SEM values; for the statistical analysis Student's t-test was used and a p value of 0.05 was considered to be significant.

Results

In-vivo secretion of cathepsin B into pancreatic juice during stimulation with caerulein

Since previous workers found that the dose of caerulein which induces the maximal rate of amlyase secretion by the in-vivo rat pancreas is approximately 0.05–0.2 μg/kg. hr and since we did not know what the equivalent dose would be in rabbits, we used 0.02–2.0 μg/kg. hr in this experiment.

The volumes of pancreatic juice obtained during stimulation with 6 different concentration are summarized in Table 1. With caerulein concentrations up to 0.5 μg/kg. hr, the volumes of pancreatic juice increased and there were significant differences between those obtained at lower concentrations (0.02–0.05 μg/kg. hr), and those obtained at highter concentrations (1.0–2.0 μg/kg. hr), on the contrary, marked reduction of pancreatic juice volumes was noted, so these highest concentra-

Table 1 Changes in volume of pancreatic juice during stimulation with 6 different concentrations of caerulein (0.02–2.0 μg/kg. hr) for 3 hours

Caerulein concentration (μg/kg. hr)	Pancreatic juice fractions (ml/kg. hr)		
	C1	C2	C3
0.02 (n=4)	0.19±0.02	0.19±0.01	0.17±0.01
0.05 (n=4)	0.25±0.02	0.23±0.01	0.22±0.02
0.2 (n=4)	1.14±0.05*	1.12±0.08*	1.10±0.06*
0.5 (n=4)	1.47±0.05**	1.52±0.05**	1.35±0.04**
1.0 (n=3)	0.63±0.09	0.58±0.08	0.50±0.07
2.0 (n=3)	0.41±0.07	0.37±0.04	0.32±0.06

(*, $p<0.01$ compared with 0.02, 0.05, 1.0, and 2.0 μg/kg. hr; **, $p<0.05$ compared with 0.2 μg/kg. hr)

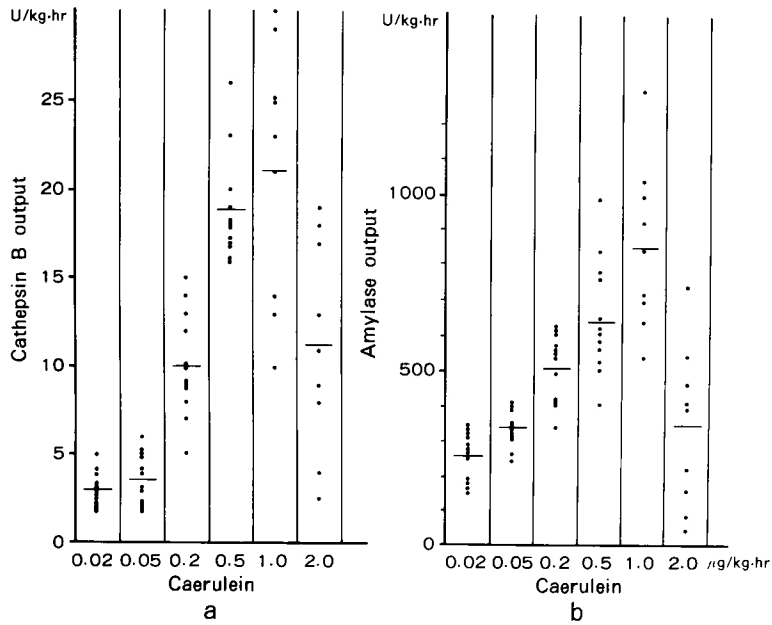

Fig 1 Cathepsin B (a) and amylase output (b) into pancreatic juice during stimulation with 6 different concentrations of Caerulein (0.02, 0.05, 0.2, 0.5, 1.0, and 2.0 μg/kg. hr)
In each group 9 or 12 hourly fractions were obtained. Horizontal rows indicate mean values.

Table 2 Changes in amylase output during stimulation with 6 different concentrations of caerulein (0.02–2.0 1mg/kg. hr) for 3 hours

Caerulein concentrations (μg/kg. hr)	Amylase output in pancreatic juice fractions (U/kg. hr)		
	C1	C2	C3
0.02 (n=4)	282±13	314±16	170±6
0.05 (n=4)	308±10	345±24	289±21
0.2 (n=4)	664±87*	585±102*	405±52*
0.5 (n=4)	741±88*	655±106*	571±56
1.0 (n=3)	861±151*	1087±169**	932±111**
2.0 (n=3)	556±125	324±150	271±139

The values are expressed as mean±SEM. (*, $p<0.05$ compared with 0.02, and 0.05; **, $p<0.05$ compared with 0.02, 0.05, 0.2, 0.5 and 2.0)

tions seem to be supramaximal doses. Both amylase output and cathepsin B output are plotted in Figure 1. For amylase output, there was the same dose-dependent increase with caerulein concentrations of up to 1.0 μg/kg. hr and significant differences between lower and higher concentrations of caerulein (Table 2), but with the highest concentration (2.0 μg/kg. hr), amlyase output was, on the contrary reduced.

Cathepsin B secretion into pancreatic juice during stimulation with caerulein, showed the same dose dependency as did amylase secretion (Table 3). At the highest concentration of caerulein (2.0 μg/kg. hr), however, cathepsin B output was inhibited as was amylase output.

The total output of amylase and cathepsin B during 3 hours showed a clearer dose-dependent

Table 3 Changes in cathepsin B output into pancreatic juice during stimulation with 6 different concentrations of caerulein (0.02–2.0 μg/kg. hr) for 3 hours

Caerulein concentrations (μg/kg. hr)	Cathepsin B output fractions (U/kg. hr)		
	C1	C2	C3
0.02 (n=4)	4±1	3±1	2±1
0.05 (n=4)	5±1	4±1	2±1
0.2 (n=4)	12±2*	11±2*	7±1*
0.05 (n=4)	20±2**	20±2**	18±2**
1.0 (n=3)	27±2†	24±5***	17±4***
2.0 (n=3)	15±3	10±4	8±3

The values are expressed as mean±SEM. (*, $p<0.05$ compared with 0.02 and 0.05; **, $p<0.05$ compared with 0.2 and $p<0.01$ compared with 0.02 and 0.05; ***, $p<0.05$ compared with 0.02, 0.05, 0.2 and 2.0; †, $p<0.05$ compared with 0.02, 0.05, 0.2 and 2.0)

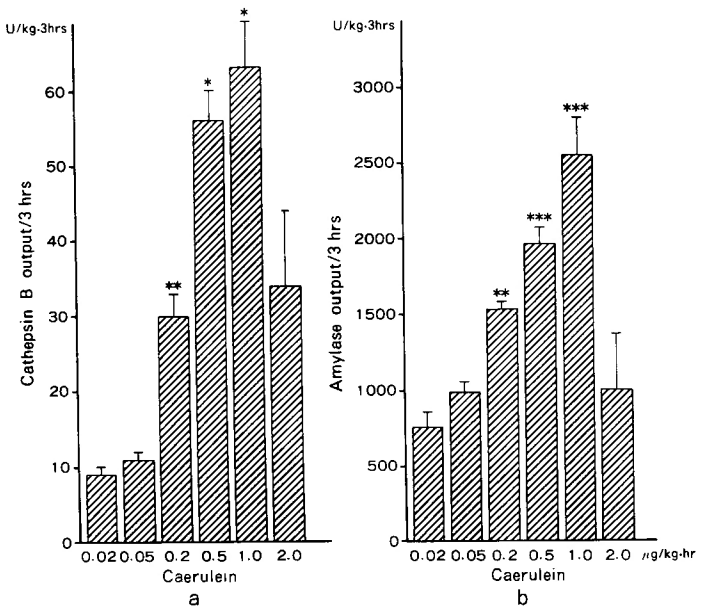

Fig 2 Changes of cathepsin B (a) and amylase output (b) into pancreatic juice during stimulation by 6 different concentrations of caerulein for 3 hours.
0.02 (n=4), 0.05 (n=4), 0.2 (n=4), 0.5 (n=4), 1.0 (n=3) and 2.0 (n=3). *: $p<0.01$ compared with 0.02 and 0.05 μg/kg. hr, and $p<0.05$ compared with 0.2 μg/kg. hr, **: $p<0.05$ compared with 0.02 and 0.05 μg/kg. hr, ***: $p<0.02$ compared with 0.02 and 0.05 μg/kg. hr, and $p<0.05$ compared with 0.2 μg/kg. hr.

relationship with the concentrations of caerulein infused (Fig. 2).

These results indicate that the secretion of cathepsin B as a lysosomal enzyme is stimulated by pancreatic secretagogue in the same manner as is that of classical pancreatic digestive enzymes, such as amylase, and the maximal output of both amylase and cathepsin B in the rabbit is obtained at caerulein concentrations of 0.5–1.0 μg/kg. hr.

At concentration of caerulein below 0.5 μg/kg. hr there was no redistribution of lysosomal en-

zyme in the subcellular fractions (Table 4);these 4 different concentrations of caerulein (0.02–0.05 μg/kg. hr) did not cause colocalization of lysosomal hydrolases and digestive enzymes. On the contrary, with higher concentrations of caerulein (1.0–2.0 μg/kg. hr), which seem to be supramaximal doses, slight redistribution of lysosomal enzyme and colocalization of lysosomal enzyme and digestive enzymes were seen (Table 4). After the above experiments were completed, the pancreatic water content and serum amylase levels were measured. There were no significant differences in pancreatic water content between the 6 groups infused with caerulein and the normal non-manipulated rabbits (Table 5). At caerulein concentrations up to 0.5 μg/kg. hr no significant difference were noted in serum amylase levels before and after the experiment, among the 4 groups, althouth the pancreatic water content and the serum amylase level in the group receiving higher concentrations of caerulein (1.0–2.0 μg/kg. hr) were slightly higher than in the other groups.

Thus, these 4 different concentrations of caerulein (0.02–0.5 μg/kg. hr) did not injure the acinar cells, but at higher concentrations (1.0–2.0 μg/kg. hr), there seemed to be a possibility of caerulein injury of acinar cells.

There was also a significant positive correlation between the amylase and the cathepsin B output

Table 4 Changes in cathepsin B activity in subcellular fractions after stimulation for 3 hours with 6 different concentrations of caerulein (0.02–2.0 μ/kg. hr) in the rabbit.

Caerulein concentrations (μg/kg. hr)	Subcellular fractionations			
	Zymogen pellet (1.0 KP)	Lysosmal pellet (12 KP)	Microsomal pellet (105 KP)	Soluble fraction (105 KS)
0.02 (n=4)	25.2±1.0	53.0±1.1	4.5±0.3	17.6±1.7
0.05 (n=4)	26.7±0.9	51.1±0.9	5.3±0.3	16.9±1.2
0.2 (n=4)	26.5±1.2	51.2±1.4	5.1±0.6	17.2±0.8
0.5 (n=4)	25.7±1.5	51.4±1.7	5.4±0.3	17.6±1.5
1.0 (n=3)	37.9±0.8*	40.2±1.4*	4.6±0.5	17.3±1.3
2.0 (n=3)	41.7±0.9*	34.3±1.2*	4.5±0.4	19.5±1.9
normal rabbits (n=4)	23.1±0.9	53.7±1.6	4.8±0.5	18.4±0.4

The values are expressed as mean±SEM. There was no significant difference between 0.02–0.5, but at higher concentrations (1.0–2.0), there was a redistribution of lysosomal enzyme. (*, $p<0.01$ compared with 0.02, 0.05, 0.2, 0.5 and normal rabbits)

Table 5 Changes in pancreatic water content and serum amylase levels after 3 hours stimulation with 6 different concentrations of caerulein (0.02–2.0 μg/kg. hr)

Caerulein concentration (μg/kg. hr)	Pancreatic water content	Serum amylase level (U/ml)	
		before catheterization	after catheterization
normal rabbits (n=4)	75±2		
0.02 (n=4)	75±2	3.5±0.2	3.7±0.2
0.05 (n=4)	76±1	3.5±0.3	3.8±0.2
0.02 (n=4)	77±1	3.5±0.3	3.8±0.3
0.5 (n=4)	76±2	3.6±0.3	3.9±0.3
1.0 (n=3)	78±3	3.6±0.2	5.8±0.5*
2.0 (n=3)	80±3	3.5±0.3	6.3±0.7*

The values are expressed as mean±SEM. (*, $p<0.05$ compared with 0.02, 0.05, 0.2, 0.5, normal animals, and before catheterization values)

into pancreatic juice during stimulation with caerulein at all the concentrations used in this experiment (Fig. 3), suggesting a direct close relationship between caerulein and the secretion of cathepsin B into pancreatic juice similar to that of amylase.

In-vivo secretion of lysosomal enzyme into pancreatic juice stimulated by intraduodenal instillation of a liquid meal

The volumes of pancreatic juice secretion of amylase, protein and cathepsin B in all the fractions before and after the intraduodenal instillation of a liquid meal are summarized in Table 6. There were no significant difference among all these fractions, although there was a slight increase after

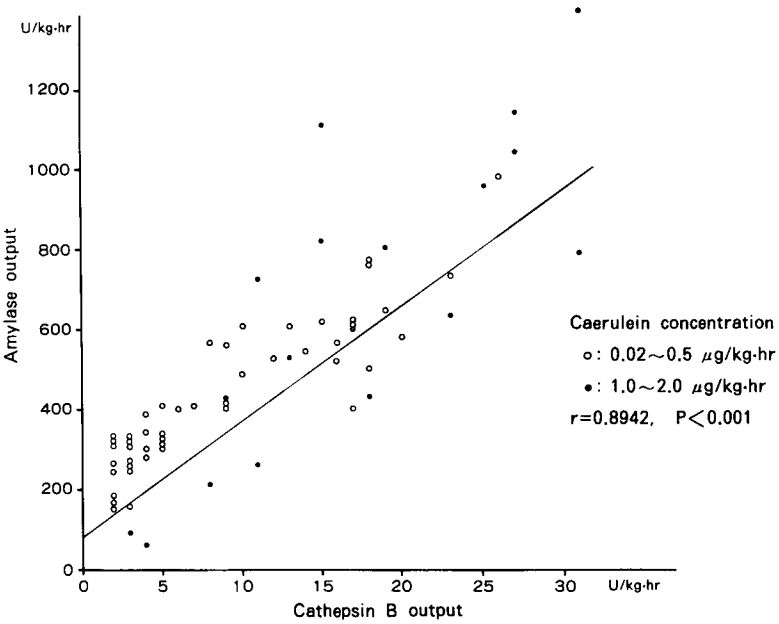

Fig 3 Correlation between amylase and cathepsin B output during stimulation with 6 different concentration of caerulein (0.02–2.0 μg/kg. hr)

Table 6 Pancreatic juice volume and secretion of amylase, protein and cathepsin B before and after intraduodenal infusion of liquid meal

	Pancreatic juice fractions						
	Pre	D1	D2	D3	D4	D5	D6
Pancreatic juice volume (ml/kg. 30 min)	0.120±0.014	0.122±0.014	0.127±0.012	0.129±0.010	0.135±0.014	0.133±0.012	0.136±0.016
Amylase output (U/kg. 30 min)	70±4	86±3*	126±8***	130±13***	105±6**	93±5*	82±7
Protein output (mg/kg. 30 min)	2.8±0.1	3.5±0.1*	4.8±0.2***	5.2±0.2***	4.5±0.1**	3.5±0.2**	2.7±0.2
Cathepsin B output (U/kg. 30 min)	not detected	0.5±0.1***	2.0±0.3***	1.1±0.2***	0.8±0.1***	not detected	not detected

There were 5 rabbits in this experiment and the values are expressed as mean±SEM. There were no significant differences among all the fractions (Pre, before meal infusion; D1–D6, after meal infusion; fractions of pancreatic juice were collected at 30-minute intervals) *$p<0.05$, **$p<0.02$, ***$p<0.01$ compared with the fraction before meal infusion (Pre).

meal infusion.

The amylase output was significantly greater after meal infusion than before meal infusion. The protein output was also significantly greater after meal infusion than before meal infusion.

These increases persisted during the 2 hours after infusion of the liquid meal, and the peak effects of meal instillation on both amylase and protein output were observed in the second and third 30-minute periods.

Cathepsin B activity was detected only after instillation of liquid meal and remained detectable for 2 hours.

When cathepsin B activity, was present there was a significant positive correlation between cathepsin B and amylase output ($\gamma=0.6455$, $P<0.01$) and between cathepsin B and protein output ($\gamma=0.6334$, $P<0.01$) in response to the intraduodenal instillation of a liquid meal.

Rabbit pancreatic duct obstruction model

Serum amylase activity was not altered within the first hour after the beginning of pancreatic duct obstruction, but thereafter, ductal obstruction caused significantly higher serum amylase activity than than that in the free-flowing group (Fig. 4) or the non-catheterized group.

Catheterization of the pancreatic duct and stimulation with secretin caused the serum amylase level to rise slightly but significantly in comparison with the level in the non-catheterized group, even when the drainage catheter was not raised to a vertical position. This latter phenomenon seems to indicate that even in its horizontal position with free-flowing pancreatic juice, the catheter itself is a mild impediment to the free flow of secretion. The infusion of secretin alone in non-catheterized control animals did not-cause a demonstrable change in serum amylase activity.

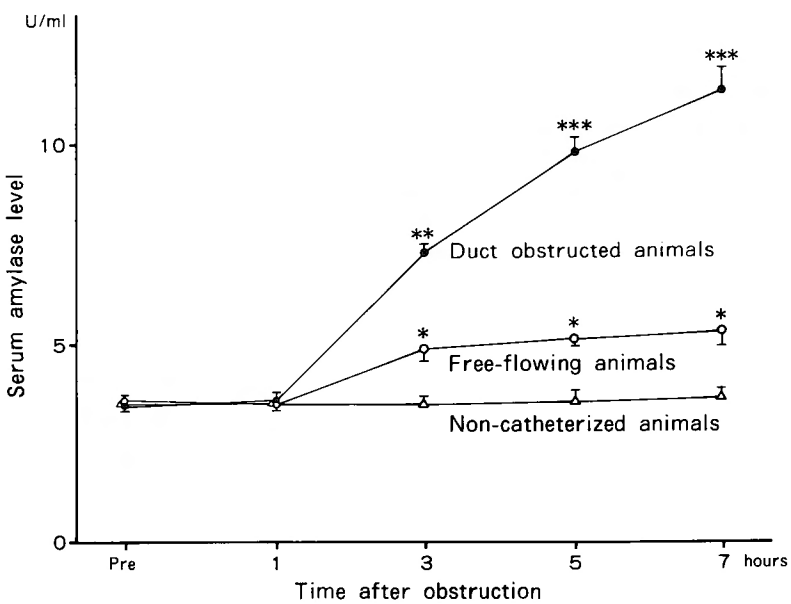

Fig 4 Changes in serum amylase levels after pancreatic duct obstruction

There were 4 rabbits in each group, and the values are expressed as mean±SEM. ●, duct-obstructed and secretin infusion group (n=4); ○, free-flowing and secretin infusion group (n=4); △, only secretin infusion group (n=4). *: $p<0.05$ compared with only secretin infusion group; **: $p<0.02$; ***: $p<0.01$ compared with the free-flowing and only secretin infusion group.

At the end of the period of obstruction, the pancreatic water content was greater than in the free-flowing and non-chatheterized rabbits, but not significantly so (Table 7). In accord with these results, redistribution of cathepsin B activity in the subcellular fractions was found in the obstructed rabbits (Fig. 5), but in the free-flowing animals no redistribution was seen in spite of a slight increase in serum amylase activity and pancreatic water content. Thus, this duct obstructed model seems to represent a mild form of acute pancreatitis and should be useful in clarifying the early events in the pathogenesis of acute pancreatitis.

Changes of amylase and cathepsin B output during stimulation with caerulein in pancreatic duct obstructed rabbits

The amylase activity in each fraction of the free-flowing group (F_1-F_7, CS_1, CS_2) is shown in Figure 6a. The output of amylase in the secretin only fractions (F_1-F_7) was less than that in the caerulein plus secretin fractions (CS_1, CS_2). The pattern of cathepsin B output in all the fractions, as shown in Figure 6b, was similar to that of the amylase output. At the end of this experiment, the free-flowing group showed no redistribution of cathepsin B activity in the subcellular fractions (Fig. 6c).

Table 7 Pancreatic water content in pancreatic duct obstructed and free-flowing animals.

Groups		Pancreatic water content (%)
duct obstructed	(n=4)	81±2
free-flowing	(n=4)	78±2
non-catheterized	(n=4)	76±2

All animals were infused with secretin (0.2CU/kg. hr) for 7 hours. The values are expressed as mean±SEM. (There were no significant differences among these three groups; the duct-obstructed group had the highest water content.)

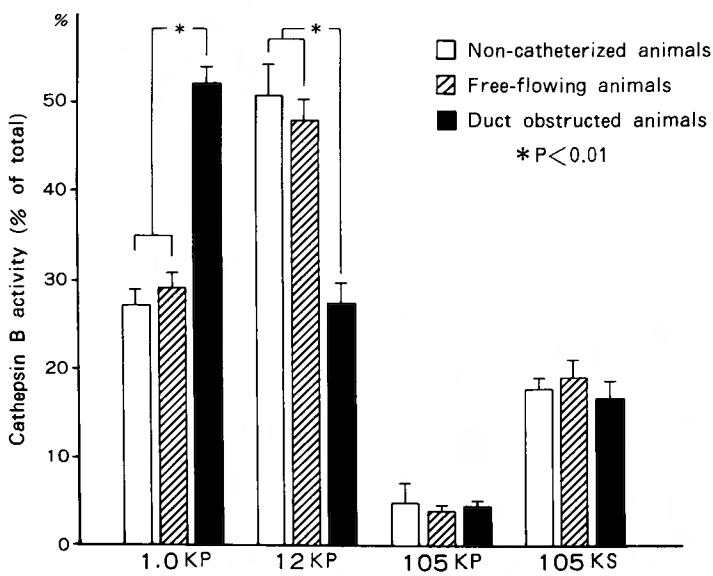

Fig 5. Changes in distribution of cathepsin B activity in subcellular fractions after pancreatic duct obstruction 1.0 KP, zymogen pellet; 12 KP, lysosome and mitochondrial pellet; 105 KP, microsomal pellet; 105 KS, soluble fraction.

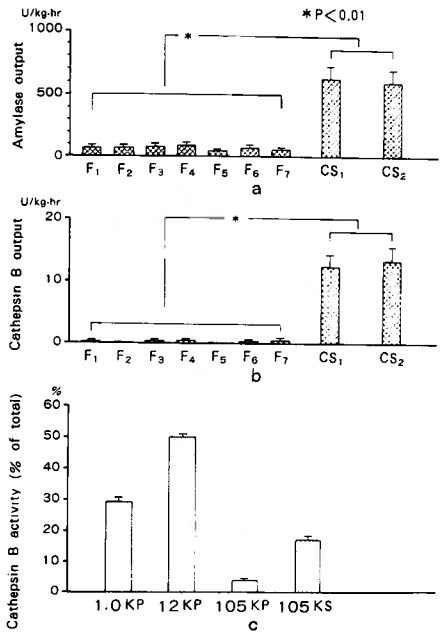

Fig 6. Changes in amylase (a), cathepsin B (b), and distribution of cathepsin B (c) activity in free-flowing animals with infusion of secretin (0.2 μg/kg. hr) for 7 hours and secretin plus caerulein (0.2 μg/kg. hr) for the next 2 hours

The values are expressed as mean±SEM of 6 rabbits. (F1–F7, secretin fractions; CS1, CS2, secretin plus caerulein fractions;) There was no redistribution of cathepsin B activity in these free-flowing animals.

These results indicate that pancreatic secretagogues such as caerulein seem to be needed to stimulate the secretion of considerable amounts of lysosomal enzymes into pancreatic juice.

After 7 hours of obstruction and secretin infusion, cathepsin B activity in the caerulein plus secretin fractions (CS_1, CS_2) was definitely and very significantly greater than that in the secretin only fractions (S_1, S_2) (Fig. 7a).

Amylase output was also very significantly greater in the caerulein plus secretin fractions than in the secretin only fractions (Fig. 7b). In addition, in the pancreatic duct obstructed group, both the amylase and the cathepsin B output during stimulation with secretin plus caerulein were significantly greater than in the non-obstructed free-flowing group (Fig. 8a, 9a). The outputs of both amylase and cathepsin B, expressed as percentages of the splenic lobe, were also significantly greater in the obstructed than in the non-obstructed animals (Fig. 8b, 9b).

These results indicate that obstruction alone does not cause a large output of cathepsin B into the pancreatic juice.

Both pancreatic duct obstruction and appropriate stimulation by a pancreatic secretagogue cooperatively seem to augment cathepsin B and amylase output into the pancreatic juice.

Furthermore, in the group which received infusions of secretin plus caerulein after 7 hours of obstruction, the redistribution of cathepsin B activity in the subcellular fraction was improved. The cathepsin B activity in the zymogen pellet (1.0 KP) was significantly lower than in the secretin only group, and in the lysosomal pellet (12 KP) it was significantly higher than that in the secretin only group (Fig. 10).

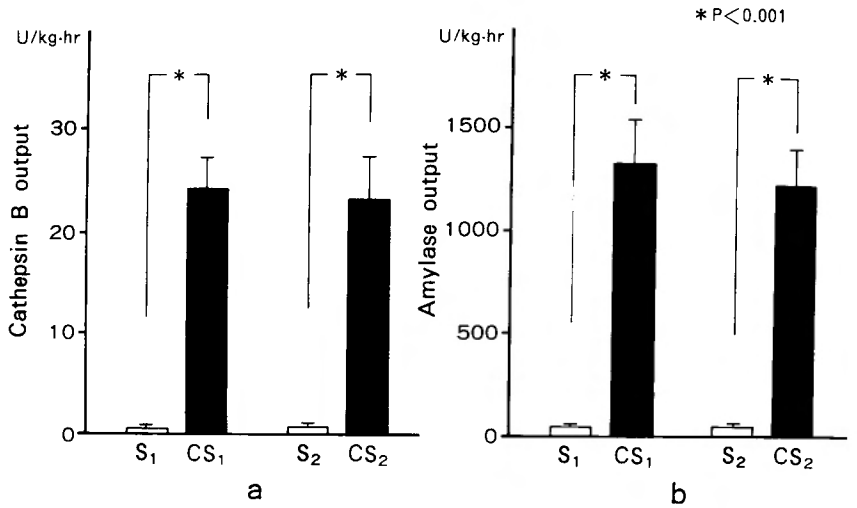

Fig 7 Changes of cathepsin B (a) and amylase (b) output into pancreatic juice after 7 hours of pancreatic duct obstruction and secretin infusion stimulated by pancreatic secretagogue for the next 2 hours
There were 5 rabbits in the group stimulated by only secretin and 8 rabbits in the group stimulated by secretin plus caerulein. The values are expressed as mean±SEM. S1, S2, secretin fractions (secretin 0.2 CU/kg. hr) (n=5); CS1, CS2, secretin plus caerulein fractions (secretin 0.2 CU/kg. hr and caerulein 0.2 μg/kg. hr) (n=8).

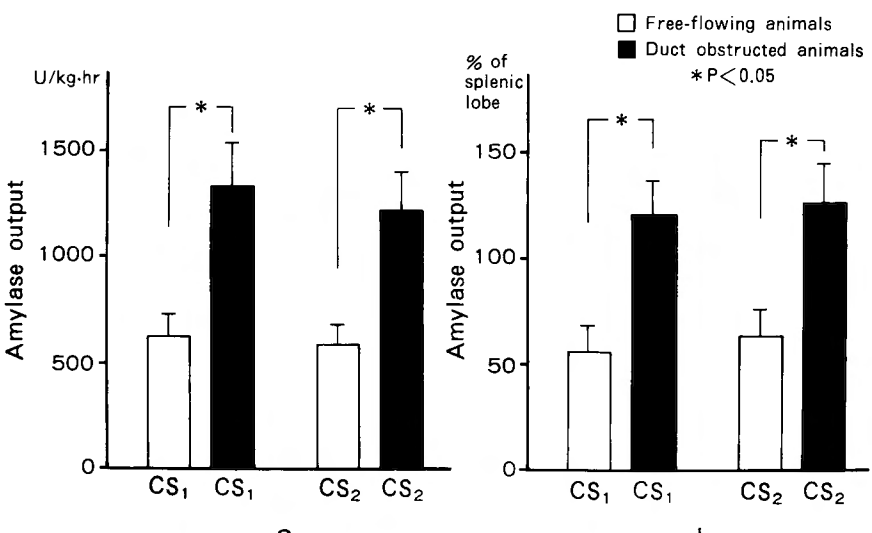

Fig 8 Changes of amylase output into pancreatic juice in duct-obstructed and free-flowing groups-during stimulation with secretin plus caerulein: amylase output (U/kg. hr) (a) and output expressed as % of splenic lobe (b) free-flowing animals (n=6); duct-obstructed animals (n=8); CS1, CS2, secretin plus caerulein fractions.

These results indicate that colocalization of lysosomal enzymes and digestive enzymes induced by pancreatic duct obstruction was improved in the animals which received caerulein by the exocytosis of both digestive enzymes and lysosomal hydrolases.

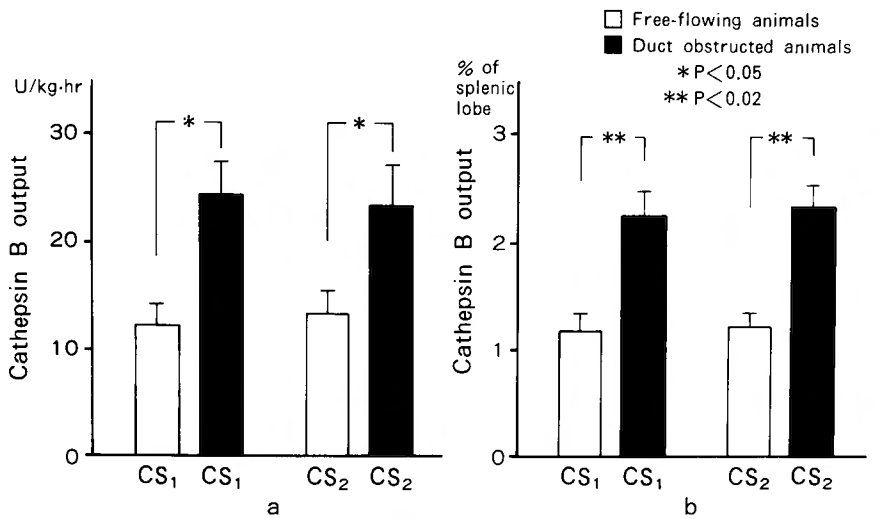

Fig 9 Changes of cathepsin B output into pancreatic juice during stimulation with secretin plus caerulein in pancreatic duct-obstructed and free-flowing animals: cathepsin B output (U/kg. hr) (a) and output expressed as % of splenic lobe (b)
There were 6 rabbits in the free-flowing group and 8 rabbits in the pancreatic duct-obstructed group. The values are expressed as mean±SEM. free-flowing animals (n=6); duct-obstructed animals (n=8); CS1, CS2, secretin plus caerulein fractions.

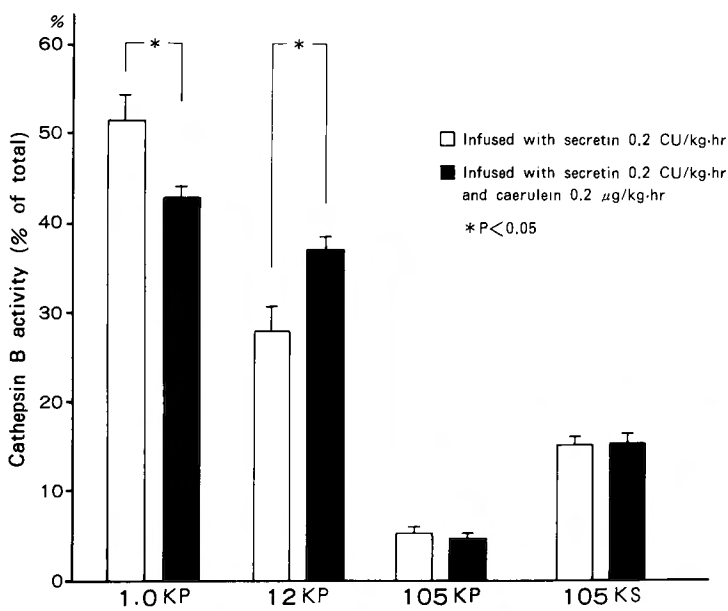

Fig 10 Changes in distribution of cathepsin B activity in subcellular fractions in pancreatic duct-obstructed groups
There were 5 rabbits in the secretin only group, and 8 rabbits in the secretin plus caerulein infused group. The values are expressed as mean±SEM.

Table 8 Weight, amylase and cathepsin B contents in splenic lobe of pancreas

	Splenic lobe (% of total)	Other portions (% of total)
weight	34.8±0.8	65.2±0.8
amylase content	40.0±1.4	60.0±1.4
cathepsin B content	37.9±0.9	62.1±0.9

The values are expressed as mean±SEM for 4 normal rabbits.

As an index of the total amylase and cathepsin B content, we used the content of the splenic lobe. In normal animals, the amylase and cathepsin B content in the splenic lobe was about 40% of the total pancreatic content (Table 8) and showed a relatively small SEM. So the splenic lobe seems to be useful as an index of the total pancreas.

Discussion

Several investigators have described in detail the cell biology of two experimental forms of acute pancreatitis, diet-induced[25,42] and secretagogue-induced[34-36,42] pancreatitis. Recently another model of acute pancreatitis in rabbits[37] has been reported: pancreatic duct obstruction and secretin infusion. Very similar phenomena were noted during the early stages of these three models, although the ultimate degree of pancreatic injury differed considerably. Although these observations may characterize the genesis of the three experimental models of pancreatitis, the relevance of the former two models to the disease in humans in not clear, since clinical acute pancreatitis, which is frequently associated with biliary tract stones, does not involve either exposure to ethionine or excessive secretagogue stimulation. Rather, gallstone pancreatitis in human appears to be precipitated by the passage of a stone through or its incarceration in the terminal portion of the common bile duct[1] The mechanism whereby such a stone might precipitate acute pancreatitis has been the subject of many studies and continues to be an issue of considerable controversy. Three hypotheses have been advocated; (a) that the stone blocks the drainage of bile and pancreatic juice into the duodenum and causes bile to reflux into the pancreatic[3,36] duct; (b) that the passage of a stone through the sphincter of Odd; renders that structure incompetent and permits activated enzymes to reflux from the duodenum into the pancreatic duct;[28] (c) that the stone, or inflammation and edema caused by its passage, results in pancreatic duct obstruction, and continued pancreatic secretion leads to ductal hypertension and rupture[29]. There have been numerous objections to each of these hypothese, not the least of which is that none of them clearly explains how activated digestive enzymes gain access to the gland parenchyma.

The studies reported in this communication may provide an important clue to the understanding of the events leading to pancreatitis, since they show that in the physiological state both pancreatic secretagogues, such as caerulein, and food intake can stimulate the secretion of lysosomal hydrolases into pancreatic juice in a dose-dependent manner as is true of classical digestive enzymes such as amylase.

Although the reason that pancreatic secretagogues and food intake stimulate lysosomal enzyme secretion into the pancreatic juice is unclear and remains to be elucidated, this bulk discharge of lysosomal hydrolases into pancreatic juice by exocytosis is the most probable mechanism of release consistent with the maintenance of normal cellular organization and suggests that lysosomal enzyme

play a physiological role in the pancreatic juice. This finding also indicates that a considerable amount of cathepsin B present in the pancreatic juice could lead to the activation of pancreatic digestive enzymes in the pancreatic duct system, since cathepsin B can activate trypsinogen, and trypsin can activate other digestive enzymes. Moreover, there is a highly positive correlation between amylase output and cathepsin B output, which leads to a few speculations: a) amylase and cathepsin B are in the same subcellular compartments in normal acinar cells, and in fact, there have been a few reports about the localization of lysosomal enzymes, such as acid phosphatase, in premature zymogen granules[21, 24]; b) lysosomes can secrete cathepsin B by exocytosis under the direct influence of caerulein; c) lysosomes may fuse with zymogen granules randomyly in the apexes of acinar cells when stimulated by caerulein. However, the amount of cathepsin B which can be secreted by stimulation with almost the maximal concentration of caerulein (0.5 μg/kg. hr) for amylase output was estimated to be only a small percentage of the total pancreatic activity within the acinar cells, and if there is colocalization of lysosomal hydrolases and pancreatic digestive enzymes in acinar cells in the normal state, it seems to be relatively small and controllable so that acinar cells are not injured.

Generally, pancreatic digestive enzymes and lysosomal hydrolases are transported separately from the Golgi apparatus to their own subcellular compartments, condensing vacuoles and lysosomes, and theoretically there is no colocalization of these two types of enzymes in acinar cells. However, at the start of the transport of these two types of enzymes, they share a common pathway from the ribosomes in the endoplasmic reticulum to the Golgi apparatus, and this mixture of lysosomal hydrolases and digestive enzymes seems to be accidental.

An earlier study using this rabbit pancreatic duct obstruction model[18] showed that pancreatic duct obstruction, under conditions in which physiological secretory pressure cannot be exceeded has dramatic effects on pancreatic acinar cell functions. Although protein synthesis continues, newly synthesized digestive enzymes are not discharged, and the digestive enzyme concentration within acinar cells increases; this phenomenon is seen in both diet-induced and secretagogue-induced acute pancreatitis in animals[12]. In both caerulein-induced[34, 35, 42] and diet-induced[25] pancreatitis, marked enlargement of zymogen-containing organelles in the cell apex and colocalization of lysosomal hydrolases and digestive enzymes within large acidic cytoplasmic vacuoles have been observed. In this study, too, subcellular fractionation experiments showed that duct obstruction leads to a redistribution of cathepsin B activity and that, as a result, lysosomal hydrolase becomes localized in a fraction that is rich in digestive enzymes. The colocalization of these two enzymes observed in our present study is probably the result of crinophagy, i.e. discharge of secretory granules into lysosomes, and a defect in the normal sorting events by which digestive enzymes and lysosomal hydrolases are separated from each other as they pass through the Golgi apparatus[8]. This colocalization phenomenon might be an important triggering event in the evolution of pancreatitis because the lysosomal hydrolase cathepsin B can activate trypsinogen and trypsin can activate other digestive enzymes. Thus, colocalization could, under appropriate coditions, result in the intraacinar cell activation of potentially dangerous digestive enzymes. Although duct obstruction might stimulate crinophagy and thus lead to the colocalization of digestive enzymes and lysosomal hydrolases by that mechanisms, these observations suggest an alternative explanation: duct obstruction, possibly by inhibiting exocytosis, might interfere with the specific events which are involved in lysosomal enzyme transport from the Golgi complex to lysosomes[18, 33, 38] and thus cause lysosomal hydrolases to be diverted into the regulated secretory pathway.

Another important findings in our present study was that lysosomal hydrolases and digestive en-

zymes, the colocalization of which is induced by pancreatic duct obstruction, are secreted when stimulated by pancreatic secretagogues such as caerulein. This findings indicates that the zymogen granules which contain lysosomal enzymes have the ability to react to pancreatic secretagogues even in this rabbit model of mild pancreatitis. This hypothesis is supported by the observation that caerulein causes redistribution of lysosomal enzyme in the apexes of acinar cells, that there seems to be less colicalization of enzymes in acinar cells after secretagogue stimulation, and that cathepsin B activity in zymogen pellets (1.0 KP) is somewhat decreased. The secretion of colocalized lysosomal hydrolases and digestive enzymes into pancreatic juice and the pancreatic duct system and the redistribution of lysosomal enzymes in the pancreatic acinar cells might have special clinical importance in the etiology of gallstone pancreatitis. Gallstone attacks are often repeated, and after the first obstruction induced by a gallstone, if the secretion of pancreatic secretagogue, such as cholecystokinin and secretin, is stimulated by food intake, colocalized digestive enzymes and lysosomal hydrolase can be secreted into the pancreatic juice together; but when the pancreatic duct system is obstructed by another stone or another attack, or if edema of the sphincter of Oddi persists, there is no way for these digestive enzymes and lysosomal hydrolases to drain into the pancreatic juice; in addition, within the acinar cells, there may be another redistribution of lysosomal enzymes. In the normal physiological state, a connection between the pancreatic ductal space and the peri acinar interstitial space has been reported [2,5] and pancreatic duct obstruction, with or without hypersecretion, seems to increase this space and to make easier the entry of digestive enzymes into the systemic circulation or the interstitium of the pancreas. Under these conditions the exocrine pancreas would be exposed to the activation of digestive enzymes by lysosomal hydrolases both within the acinar cells, where lysosomal hydrolses are colocalized in zymogen granules, and outside the acinar cells, where lysosomal and zymogen proteins are colocalized in the pancreatic duct system and pancreatic interstitium. These circumstances would lead to ductal hypertension and damage to the protective barrier of ductal epithelium[17] induced by simple mechanical pressure or by the influx of infected biliary juice sometimes found in choleithiasis, and the pancreas would be more susceptible to autodigestion both inside and outside the acinar cells.

In clinical and experimental models of acute pancreatitis (diet-induced, secretagogue-induced, or pancreatic duct obstruction), this disease seems to have a rather broad spectrum, and it is accepted that edematous pancreatitis can, under appropriate conditions such as hemorrhagic shock[30], progress to the more severe forms of pancreatitis, characterized by hemorrhage and/or necrosis of the gland.

Other potential modulators of the severity of pancreatitis are alterations in the pancreatic microcirculation[7] and changes in pancreatic ductal permeability induced by oral agents[43], which might permit pancreatitis to progress to a more severe form.

Although the factors responsible for such a progress are not yet clearly understood, the secretion of lysosomal hydrolases, which can potentially activate pancreatic digestive enzymes, into the pancreatic juice in bulk when appropriately stimulated by pancreatic secretagogues seems to favor the conversion of the mild edematous form into severe hemorrhagic and necrotic pancreatitis and is a possible trigger of pancreatitis in the "common channel" theory.

Our pancreatic duct obstruction model provides a very mild type of pancreatitis in its broad spectrum. Although it has the very slight defect that catheterization of the pancreatic duct and secretin infusion perse cause a slight rise in serum amylase levels, this increase was less than that occuring in other experimental models of pancreatitis which show redistribution of lysosomal enzymes or increased pancreatic water content.

This model seems to be very useful in clarifying the very early events in acute pancreatitis, in spite of the minor side effects due to catheterization of the pancreatic duct.

We now have three different animal models of expreimental pancreatitis. The mildest is our present model of pancreatic duct obstruction, the secound is secretagogue-induced pancreatitis, and the most severe is diet-induced pancreatitis. The three models seem to represent the different degrees of severity in the broad spectrum of acute pancreatitis, but each has the same phenomenon of redistribution of lysosomal hydrolases and colocalization of lysosomal enzymes and digestive enzymes. It is very useful to have these three models to determine the factors which effect the severity of this disease.

Although the currently reported studies support the hypothesis that ductal obstruction may be important in the pathogensis of gallstone pancreatitis, it is claer from these as well as many other studies that ductal obstruction alone is not sufficient to cause the morphological changes of pancreatitis.

Clearly, other events must occur if the changes induced by ductal obstruction are to lead to more severe pancreatic injury. Studies designed to identify and clarify those events are of great importance, because they are likely to be the ultimate determinants of the severity of pancreatitis, and advance our knowledge of the pathogenesis and pathophysiology of this disease. Even more important, they may lead to therapeutic advances.

In this study, we cannot explain clearly the significance of the secretion of lysosomal enzymes into pancreatic juice during stimulation by pancreatic secretagogues or food intake, but our recent study showed that other lysosomal enzymes including arylsulfatase, N-Acetyl-β-D-glucosaminidase, β-D-glucosamidase, and leucine naphthylamidase, were secreted into pancreatic juice during stimulation by caerulein.

Although these findings strongly suggest that exocysis of lysosomes is induced by gut hormones, their pathophysiological roles in the pancreatic juice remain to be investigated.

The model of controlled pancreatic duct obstruction described here is expected to be of considerable value in future studies designed to address that issue, since it is a method of minimizing the severity of the disease.

Acknowledgements

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和文抄録

生理学的および病態下での家兎膵液中へのライソゾームおよび膵消化酵素の分泌

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生理学的および病態下での膵外分泌刺激ホルモンによる膵液中へのライソゾーム酵素分泌を検討する目的にて，セルレインの6種の用量 (0.02, 0.05, 0.2, 0.5, 1.0, 2.0 μg/kg. hr) の投与下家兎膵液中へのカテプシンBの分泌量とともに，膵管へのカテーテルを7時間にわたり閉塞（同時にセクレチンを 0.2 CU/kg/h にて投与）した後の分泌量の変化を検討した．さらに，十二指腸内への食餌 (2 g/kg) 投与のライソゾーム酵素の膵液中への分泌に及ぼす影響も検討した．セルレインは膵酵素アミラーゼと同様にカテプシンBの膵液中への分泌を誘起するとともに，カテプシンB分泌量とアミラーゼ分泌量との間には有意な正の相関関係が存在した．7時間の膵管閉塞にて，血清アミラーゼ値の有意な上昇とともに，膵腺房細胞の分画法により，カテプシンB活性の再分布，つまりチモーゲン分画での活性の上昇が観察された．これらの変化は，食餌誘起性膵炎やセルレイン誘起性膵炎の初期での観察に合致するものであり，膵消化酵素とライソゾーム酵素の共存を示すものと考えられた．さらに，十二指腸内への食餌負荷もアミラーゼとともにカテプシンB分泌を誘起した．また，膵管閉塞後には，セルレイン刺激下でのカテプシンB分泌量がコントロール群に比べ有意に増加した．これらの結果は，生理学的状況下でのライソゾーム酵素の膵液中への分泌を示すとともに，膵管閉塞後のライソゾームと共存したチモーゲン顆粒の膵液中への分泌も示した．これらの結果はまた，膵液中でのライソゾーム酵素の生理学的役割を示唆させるとともい，この膵液中へのライソゾーム酵素の分泌が，膵腺房細胞の正常な機能を維持する上で重要な機構であることも示唆させた．